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BIOCHEMICAL CHANGES AND THEIR REGULATION DURING SPORE FORMATION--ETC(U)
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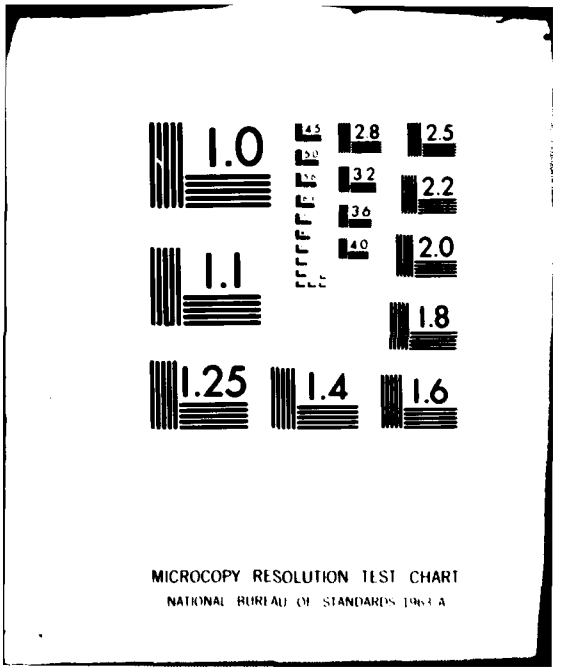
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BIOCHEMICAL CHANGES AND THEIR REGULATION DURING SPORE FORMATION AND GERMINATION

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Final Technical Report

Peter Setlow

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Department of Biochemistry
University of Connecticut Health Center
Farmington, Connecticut 06032

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number):

Metabolism of several important small molecules has been examined during sporulation and spore germination in Bacillus megaterium. These studies have indicated (1) The major known low molecular weight thiol/disulfide in Bacillus species is Coenzyme A (CoA). (2) CoA in growing or sporulating cells is in either an acyl form or as the free thiol, but that in dormant spores ~ 75% is in a disulfide form with 50% in disulfide linkage to spore core proteins; these disulfides are cleaved in the first minutes of spore germination. (3) Dormant spores contain an NADH-linked disulfide reductase which cleaves CoA disulfides.

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This enzyme is low or absent from log-phase or early sporulating cells, and appears during sporulation. It has no activity on cystine, glutathione or pantethine, and has highest activity on 4',4"-phosphopantethine. 4) Bacillus megaterium cells contain a very low level of cyclic GMP (cGMP), but cGMP is not found in spores and it appears unlikely to be a modulator of sporulation, germination or outgrowth. 5) The pH within dormant spores is ∿ 6.3, a value which is independent of the external pH. However, early in spore germination the internal pH rises to 7.5. 6) The key enzyme in regulation of 3-phosphoglycerate (3-PGA) accumulation during sporulation, and its rapid utilization during spore germination (and thus ATP production) is PGA mutase. This enzyme, as well as enolase have been purified to homogeneity from cells and spores, and the enzymes from both stages of growth appear identical. 7) The 3-PGA mutase has an absolute and specific requirement for Mn^{++} for activity, and evidence was obtained that the regulation of this enzyme *in vivo* is accomplished at least in part by regulation of levels of free Mn^{++}. 8) Catabolism during spore germination of the dormant spore's depot of 3-PGA proceeds to acetate. Much of the alanine generated by proteolysis during germination is also catabolized to acetate. Thus catabolism of endogenous reserves during spore germination generates ATP, NADH and acetyl-CoA. 9) The low-molecular weight proteins unique to bacterial spores were localized to spore DNA by studies involving cross-linking of the proteins to DNA with ultraviolet light. This is further support for a role for these unique proteins in spore radiation resistance.

Final Technical Report

The objectives of this project, as given in the original grant application, were as follows.

"Metabolism of several important small molecules will be studied during sporulation and spore germination in several bacterial species. Investigations will focus on the first minutes of the initiation of spore germination, and the period in sporulation between the appearance in the mother cell of a forespore and the conversion of the latter to a dormant spore. During the latter process special attention will be given to distinguishing between events in the mother cell and in the developing forespore. Metabolism of pyridine nucleotides and low molecular weight thiols and disulfides will be studied in both sporulation and germination, especially with regard to the following questions: 1) what are the levels and oxidation states of these compounds; 2) what are the regulatory mechanisms and the enzymes involved in causing any changes in the levels or oxidation states of these compounds; 3) what are the sources of reducing power in the dormant spore which are utilized in the first minutes of germination; and 4) what is the importance of generation of reducing power for the initiation of spore germination? Metabolism of adenosine triphosphate (ATP) and 3-phosphoglyceric acid (3-PGA) will be studied only during sporulation in the hopes of answering the following question: how are metabolism and, therefore, enzymes regulated in the mother cell and forespore in order to allow the massive accumulation of 3-PGA within the developing forespore followed by conversion of ATP to AMP?

Although data from the above studies will be significant of itself, this data should also permit the identification of several spore enzymes which fulfill the following requirements: 1) the enzyme is present in the dormant spore along with high levels of its substrate(s), yet there is no reaction in the dormant spore; 2) the enzyme acts rapidly on its substrate in the first minutes of germination; and 3) the kinetics of the appearance of enzyme and its substrate during sporulation is such that the enzyme must be maintained in an inactive form in the developing forespore to allow accumulation of its substrate. Possible enzymes of pyridine nucleotide, thiol or 3-PGA metabolism which might fulfill these criteria are phosphoglycerate mutase, a disulfide reductase or a dehydrogenase. Other enzymes which are known to fulfill the first two criteria are arginase, glutamate decarboxylase and the protease degrading the high levels of unique spore proteins (A and B proteins) during germination. The enzymes which fulfill all three criteria will be purified and examined during sporulation, germination, in the spore and in the test tube in order to determine what mechanisms are or could be utilized to maintain them in an inactive form in the dormant spore. Possible mechanisms which will be studied will include: 1) compartmentation; 2) the environment of the spore (anhydrous or low water activity); 3) presence of specific non-covalent inhibitors; and 4) enzyme inactivation by covalent modification."

During the three year period covered by this report, many of the studies outlined above have been completed, and some new areas of research have been initiated and/or completed. Achievements in the three year period are given

below. Most of the work utilized Bacillus megaterium, but other species have given similar data when studied.

Thiol/Disulfide status - Work prior to the initiation of this project demonstrated that dormant spores lacked NADH and NADPH, although the oxidized forms of these coenzymes were present (1). Both NADH and NADPH disappeared from the developing spore late in sporulation, but the reduced forms were generated early in germination, and using endogenous sources of reducing power if necessary.

Given these findings, the metabolism of low-molecular weight thiols and disulfides was then studied. Early studies showed that spores of several Bacillus species contained no significant amount of cysteine/cystine or reduced or oxidized glutathione (2). Similarly cells of B. cereus and B. megaterium contained no detectable glutathione, and the major low molecular weight thiol/disulfide identified was Coenzyme A (CoA). Dormant spores of Bacillus megaterium were found to contain $\sim$ 850 pmol of CoA per milligram of dry weight. Of this total, less than 1.5% was acetyl-CoA, 25% was CoA-disulfide, 43% was in disulfide linkage to protein, and the remainder was the free thiol. Dormant spores of Bacillus cereus and Clostridium bifermentans contained 700 and 600 pmol of CoA per milligram of dry weight, respectively; in both species $\sim$ 45% of the CoA was in disulfide linkage to protein. During germination of spores of all three species, > 75% of the CoA-protein disulfides were cleaved. In B. megaterium, cleavage of these disulfides during spore germination did not require exogenous metabolites and occurred at about the same time as the initiation of germination. Much of the CoA was converted to acetyl-CoA at this time. Dormant spores also contained reduced nicotinamide adenine dinucleotide-dependent CoA-disulfide reductase at levels higher than those in other stages of growth. The level of total CoA in growing cells was two- to three-fold higher than in spores. This level remained constant throughout growth and sporulation, but < 2% of the total cellular CoA was in disulfide linkage to protein until late in sporulation. The CoA-protein disulfides accumulated exclusively within the developing spore at about the time when dipicolinic acid was accumulated. The function of these CoA-protein disulfides is not yet clear, but they could be involved in the spore's dormancy (by blocking key enzyme-SH groups) or heat resistance (by protecting protein-SH groups).

Further studies on the disulfide reductases from spores have led to the identification of an NADH-linked disulfide reductase which is specific for disulfides containing 4',4"-phosphopantethine (3). This enzyme has no activity on cystine, glutathione or pantethine, but will cleave CoA-S-S CoA and 4',4"-phosphopantethine. The latter is the best substrate, but its K_m is 0.7 mM (for CoA-S-S-CoA it is 20 mM) suggesting that we have not yet found the true in vivo substrate for this enzyme. The enzyme is low or absent from log phase or young sporulating cells, and appears only during sporulation, reaching its highest level in the dormant spore. The enzyme has been partially purified and characterized. The general properties of this enzyme suggest that it might be involved in the formation and cleavage of the CoA-protein disulfides described above. However, more work is needed to prove this point.

3-PGA metabolism - Initial work on regulation of 3-PGA catabolism during sporulation and spore germination centered on the glycolytic enzymes enolase and PGA mutase (4,5).

A simple two-step procedure for purification of enolase from germinated spores or vegetative cells of Bacillus megaterium was described. The procedure resulted in a 1,200-fold purification with production of homogeneous enzyme in ~ 75% yield; the enzymes from spores and cells seemed identical. The molecular weight of the native enzyme was 335,000, with a subunit molecular weight of 42,000. The enzyme required Mg^{2+} and was inhibited by ethylenediaminetetraacetic acid and fluoride ions. The Michaelis constants for 2-phosphoglyceric acid and Mg^{2+} were 7.1×10^{-4} and 4.7×10^{-4} M, respectively.

Phosphoglycerate phosphomutase was purified to homogeneity from vegetative cells and germinated spores of Bacillus megaterium, and the spore and cell enzymes appeared identical. The enzyme is a monomer of molecular weight 61,000. The compound 2,3-diphosphoglyceric acid is not required for activity, but the enzyme has an absolute and specific requirement for Mn^{2+}. The enzyme is inhibited by ethylenediaminetetraacetate and sulfhydryl reagents, has a pH optimum of about 8.0, and has K_m values for 3-phosphoglyceric acid and Mn^{2+} of 5×10^{-4} and 4×10^{-5} M, respectively.

Detailed studies of the nature of the PGA in dormant and germinated spores then led to the identification of PGA mutase as the key enzyme for regulation of PGA metabolism (6,7). The large depot of phosphoglyceric acid (PGA) which is accumulated within spores of Bacillus megaterium is > 99% 3-phosphoglyceric acid (3-PGA). The 3-PGA depot is stable in forespores and dormant spores, but is utilized rapidly during spore germination. When spores were germinated in KBr plus NaF, the PGA depot was not utilized, but 13% of the 3-PGA was converted to 2-PGA. These data suggest phosphoglycerate phosphomutase as the enzyme which is regulated to allow 3-PGA accumulation during sporulation. Young isolated forespores, in which 3-PGA was normally stable, utilized their 3-PGA rapidly when incubated with Mn^{2+} plus the divalent cation ionophore X-537A; Mn^{2+} or ionophore alone or Mg^{2+} or Ca^{2+} plus ionophore was without effect. Young forespores contained significant amounts of Mn^{2+}. However, forespore Mn^{2+} exchanged slowly with exogenous Mn^{2+} and was removed poorly by toluene treatment. This suggests that much of the forespore Mn^{2+} is tightly bound to some forespore component. Since phosphoglycerate phosphomutase from B. megaterium has an absolute and specific requirement for Mn^{2+}, these data suggest that the activity of this enzyme in vivo may be regulated to a large degree by the level of free Mn^{2+}. Indeed, the activity of this enzyme in forespore or dormant spore extracts was stimulated > 25-fold by Mn^{2+}, whereas comparable extracts from cells or germinated spores were stimulated only two- to fourfold.

Analysis of extracts of dormant spores has revealed no inhibitors of PGA mutase other than compounds which inhibit by competing for the necessary metal-Mn^{++}. Consequently identification and analysis of possible Mn^{++} chelators (other than dipicolinic acid) is in progress. Presumably these other chelators would be destroyed and/or inactivated early in germination thus releasing the Mn^{++} required to activate PGA mutase at this time.

The model proposed from the work cited above suggests that levels of free Mn^{++} play a role in regulating PGA mutase and thus 3-PGA metabolism. This then would regulate ATP metabolism in sporulation and germination. This type of regulation may also exist for the specific spore protease, which requires Ca^{++}

to maintain its active conformation. Interestingly, there are now several reports that low levels of free divalent ions are involved in dormancy in other systems, and that breaking of dormancy (i.e. germination), is accompanied by (or can be caused by) increased levels of free divalent ions (8,9). Thus regulation of dormancy at least in part by levels of free divalent ions may be a general phenomenon in biology.

Other areas of work - Several other topics were also investigated during the grant period, although the effort expended on them was less. These areas included

Acetate production during spore germination (10) - When Bacillus megaterium spores germinate in the absence of an exogenous carbon source, the first minutes of germination are accompanied by production of large amounts ($\sim$ 70 nmol/mg of dry spores) of acetate and much smaller amounts of pyruvate and lactate. The majority of these compounds are excreted into the medium. Exogenous pyruvate and alanine are also converted to CO_2 and acetate by germinating spores, presumably by using the pyruvate dehydrogenase that is present in dormant spores. These data suggest that the 3-phosphoglyceric acid stored in the dormant spore and the alanine generated by proteolysis early in germination can be catabolized to acetate during germination with production of large amounts of reduced nicotinamide adenine dinucleotide, acetyl coenzyme A, and adenosine 5'-triphosphate.

Cyclic GMP levels in B. megaterium (11) - Previous work has shown that B. megaterium contains no cyclic AMP. However, no data for cyclic GMP (cGMP) was available. Consequently a study of the level of this potentially important regulatory molecule was undertaken. The level of cyclic GMP was less than one molecule per organism in dormant, germinated, and outgrowing spores of Bacillus megaterium. A significant level ($\sim$ 8 pmol/g, dry weight) of cyclic GMP was found in early to mid-log phase cells, but the level fell to below 0.2 pmol/g, dry weight, in late-log phase and only rose slightly to $\sim$ 0.9 pmol/g, dry weight, in stationary phase. No significant amount of cyclic GMP was detected in the growth medium at any time. This data suggests that cGMP is not involved in the regulation of sporulation, germination or outgrowth.

Localization of basic spore proteins (12) - Two low-molecular-weight basic proteins, termed A and B protein, comprise about 15% of the protein of dormant spores of Bacillus megaterium. Irradiation of intact dormant spores with ultraviolet light results in covalent cross-linking of the A and B proteins to other spore macromolecules. The cross-linked A and B proteins are precipitated by ethanol and can be solubilized by treatment with deoxyribonuclease (75%) or ribonuclease (25%). Irradiation of complexes formed in vitro between deoxyribonucleic acid (DNA) or ribonucleic acid and a mixture of the low-molecular-weight basic proteins from spores also resulted in cross-linking of A and B proteins to nucleic acids. The dose-response curves for formation of covalent cross-links were similar for irradiation of both a protein-DNA complex in vitro and intact spores. However, if irradiation was carried out in vitro under conditions where DNA-protein complexes were disrupted, no covalent cross-links were formed. These data suggest that significant amounts of the low-molecular-weight basic proteins unique to bacterial spores are associated with spore DNA in vivo. Consequently it is possible that the association of these proteins with spore DNA is involved in the resistance of spore DNA to damage by ultraviolet light.

Low pH within the spore (13) - Measurements of the distribution of the permeant weak base methylamine have shown that the pH within the dormant spore's core is 6.3. This value is not increased by heat shock or by soaking spores in media of higher (7.5-9) pH. However, the internal pH rises rapidly to 7.5 upon spore germination. Since spore enzymes such as the PGA mutase and the specific spore protease have pH optima of 7.5-8, it is possible that the dormant spore's low internal pH is a contributing factor in its metabolic dormancy. In addition, knowledge of this parameter will allow the use of the correct conditions for in vitro experiments designed to duplicate in vivo conditions.

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